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Validation of SSR markers for identification of high-yielding and *Phytophthora Capsici* root rot resistant chilli genotypes

Tazien Bukhari[1], Rashid Mehmood Rana[1✉], Azeem Iqbal Khan[2], Muhammad Azam Khan[3], Atta Ullah[4], Misbah Naseem[1], Humaira Rizwana[5], Mohamed S. Elshikh[5], Muhammad Rizwan[6✉] & Rashid Iqbal[7,8✉]

The study was designed to validate the previously reported 34 SSR markers using 78 chilli genotypes to detect significant trait specific markers as well as superior genotypes resistant to *Phytophthora capsici* root rot (PcRR). In this context, the identification of germplasm with higher yield per plant (YPP) leads to hype in stress tolerance index (STI) in genotypes, Chakwal3 (11.98), Greenfire (10.14), Advanta5017 (9.94) and Chakwal4 (7.8). The identified genotypes were also found as resistant and moderately resistant due to existence of below 50% of disease incidence. Moreover, biplot showed the interrelation of STI with YPP through the formation of acute angle by their respective vectors. In the current study, the markers Hpms1172 and CAMS177 was found significant for STI. However, the marker CAMS066 was found associated with relative cell injury, CA06g27450 with disease incidence and CAMS173 with relative leaf damage. The bright bands on gel pictures of significant markers showed the association of these markers with resistant genotypes i.e. Chakwal3, Advanta-5017 and Chakwal4 as well as with a single moderately resistant genotype i.e. Greenfire. The markerstudes confirmed the phenotypic data by showing association of markers i.e. Hpms1172 and CAMS177, r with stress tolerance index. The principal coordinate analysis aligned with the results obtained from marker-assisted selection. Thus, currently practiced marker assisted selection detected high yielding genotypes in PcRR disease stress condition that will be helpful in progressing breeding programs in chilli.

Chilli is a versatile vegetable crop used extensively as spice, pharmaceutical product, ornamental plant, ingredient in cosmetics, weapon, and biopesticide[1]. Globally, China is the largest producer of green chilli, while India leads in dry chilli production. Worldwide, green chilli is grown on 2.0 Mha (million hectare) of area while dry chilli is on 1.6 Mha of area. Worldwide production of green and dry chilli is 36.2 MT (million tons) and 4.8 MT with the yield of 17.6 and 2.9 tons per hactare, respectively[2]. Globally Pakistan stands among the top ten producers of dry chilli[2]. According to the economic survey of Pakistan 2022-23, chilli crop is cultivated under 0.03 Mha of area with 0.08 MT of production that shares 1.5% in country 's GDP (Gross domestic product)[3].

Chilli, as one of the most highly demanded condiment, has inspired chilli breeders worldwide to continually develop and improve chilli varieties for increased yield. However, chilli yield is often hindered by biotic and abiotic factors. Among biotic stresses, the root rot caused by *Phytophthora capsici*, has become a serious limitation in chilli production around the world, often leading to complete yield loss[1]. It is the 5th highly destructive oomycete

[1]Department of Plant Breeding and Genetics, PMAS-Arid Agriculture University, Rawalpindi 46300, Pakistan. [2]Department of Plant Breeding and Genetics, University of Agriculture Faisalabad, Faisalabad 03802, Pakistan. [3]Department of Horticulture, PMAS-Arid Agriculture University, Rawalpindi 46300, Pakistan. [4]Department of Plant Pathology, Agriculture Research Institute, Mingora, Swat 19200, Pakistan. [5]Department of Botany and Microbiology, College of Science, King Saud University, P.O. 2455, Riyadh 11451, Saudi Arabia. [6]Institute of Crop Science and Resource Conservation (INRES), University of Bonn, 53115 Bonn, Germany. [7]Department of Agronomy, Faculty of Agriculture and Environment, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan. [8]Department of Life Sciences, Western Caspian University, Baku, Azerbaijan. ✉email: rashid.pbg@uaar.edu.pk; m.rizwan@uni-bonn.de; rashid.iqbal@iub.edu.pk

that once entered into soil can survive more than 10–20 years due to its broad host range, having dormant and motile zoospores and is difficult to control through cultural practices[4]. The causal organism *Phytophthora capsici*, flourishes on wet soil with 80–100% moisture, on stagnant water, and on 20–30 °C of temperature[5]. An attack by *Phytophthora* on chilli plants leads to damping-off lesions at basal part of the stems, which eventually cause stem and root girdling, resulting in the sudden wilting of leaves[6]. Invasion of pathogens triggers the excessive production of reactive oxygen species (ROS) through mitochondria, chloroplasts, and peroxisomes of host plant, causing damage to the plant's macromolecules[7]. Initially, ROS serves as an antimicrobial signal in response to the pathogen-host interaction, known as oxidative burst[8,9]. Latterly, ROS damages the cell membrane, disrupts redox balance that leads to the production of malondialdehyde and lipid peroxides. This process contributes to membrane damage and programmed cell death (PCD) that ultimately resulting in appearance of necrosis spots on host cells[10,11].

The management of *Phytophthora capsici* is challenging because of its broad host range, soil-borne characteristics, and unpredictable mating habits. Moreover, its control is complicated due to the existence of 45 physiological races of *Phytophthora*, which require distinct R genes including Snakin-1[12], CaPhyto[13], CaDMR1[14] etc. Resistance in *Capsicum* to *Phytophthora capsici* root rot (PcRR) is genetically and physiologically complex with reports of single, two and multiple gene system being involved[15]. It is necessary to develop strategies to control PcRR and to understand the genetics of plant-pathogen interaction. Therefore, developing varieties carrying effective resistance against destructive pathogens has become a priority for breeders. This varietal development can be attained through phenotypic and genotypic characterization of crop.

The reliability of phenotypic screening is often questioned due to the significant influence of environmental factors, which lead to unstable and false positive selections[16,17]. Therefore, the use of molecular markers is proved as a powerful alternative that helps in perfection of genotypic screening due to its environment free impact. Resistance to Phytophthora in chilli has previously been assessed through the identification of molecular markers, such as SSR markers: CAMS 405, Hpms 1–62, CAMS 839, HpmsE034 etc., CAPS markers: ASC037, ASC031 and SCAR marker: ASC035p[18–20]. The validation of tightly linked molecular markers using marker assisted selection (MAS) method is the prerequisites for the successful selection of resistant genotype[21]. The marker assisted selection was previously reported by Alsaleh et al., where they detected wheat genotypes having low Cadmium content[22]. Due to the limited number of existing studies, this research was designed to identify strongly associated markers by validating 34 previously detected SSR markers and to select high-yielding, disease-resistant genotypes using a marker-assisted selection (MAS) approach for Phytophthora capsici root rot (PcRR) resistance in chilli[23]. The identified markers will facilitate in the transfer of disease-resistant genes in superior chilli genotypes, but these will also aid in identification of *Phytophthora* resistant genotypes.

Results

Phenotypic assessment of chilli germplasm

The seventy-eight chilli genotypes were assessed using data obtained from characterization of eight phenotypic traits viz. YPPc; yield per plant under the control condition (g), YPPi; yield per plant under the infected condition (g), STI; stress tolerance index, RCI; relative cell injury (%), CV; cell viability (%), DI; disease incidence (%), DSI; disease severity index (%) and RLD; relative leaf damage (%) for PcRR resistance. The obtained data were further subjected to ANOVA and principal component analysis (PCA).

Yield per plant

The analysis of variance for yield per plant displays the significance in both conditions (Table 1). Moreover, YPPc was ranged from 2.10 g (15/4) to 98.61 g (Chakwal3) with mean value of 26 g, however, YPPi was observed with 1.76 g (15/4) to 90.69 g (Chakwal3) range of values, with mean of 24 g value (Table 1). Furthermore, the mean performance of chilli genotypes was measured by comparing adjusted means of genotypes with least significant increase (LSI) based means of checks (Supplementary Table S5 online). The LSI based mean value of superior check Chakwal3 depicted the maximum yield per plant under control condition (mean $_{check}$ +LSI $_{check}$=99.01 g) when compared to the adjusted values of other chilli germplasm. Similarly, on comparison with

	Df	YPPc	YPPi	STI	RCI	CV	DI	DSI	RLD
Block unadjusted	3	1895	1246	19.8	276.63	700.97	390.98	733.2	383.01
Genotypes adjusted	77	610**	533**	10.8**	402.55**	563.87***	963.11**	1183**	232.11**
Control	1	1585**	2472**	34.9**	1027.3**	442.98 ***	262.89**	6194.4**	509.16**
Control vs. augmented	76	597**	507**	10.4**	396.76**	565.46***	972.32**	1117.1**	228.46**
Residuals	3	17	9	0.12	75.62	0.37	0.32	0.8	0.78
Coefficient of variation		16%	12%	3.1%	13.9%	1.2%	0.7%	2%	6%
Range		2.10-98.61	1.76–90.69	0.01–11.98	23.16–88.56	16.11–93.96	6.81–100	1.12–100	1.10-89.45
Means		26	24	2.2	66.06	52.34	78.66	44.77	15.22
Least significant increase		19.34	10.24	0.14	3.41	0.42	0.36	0.91	0.89

Table 1. The analysis of variance and descriptive statistics for seventy-eight Chilli genotypes. YPPc(g) = yield per plant under control condition; YPPi(g) = yield per plant under infected condition; STI = stress tolerance index; RCI = relative cell injury; CV(%) = cell viability; DI(%) = disease incidence; DSI(%) = disease severity index; RLD(%) = relative leaf damage; Significance codes:'**= significant; NS = non-significant.
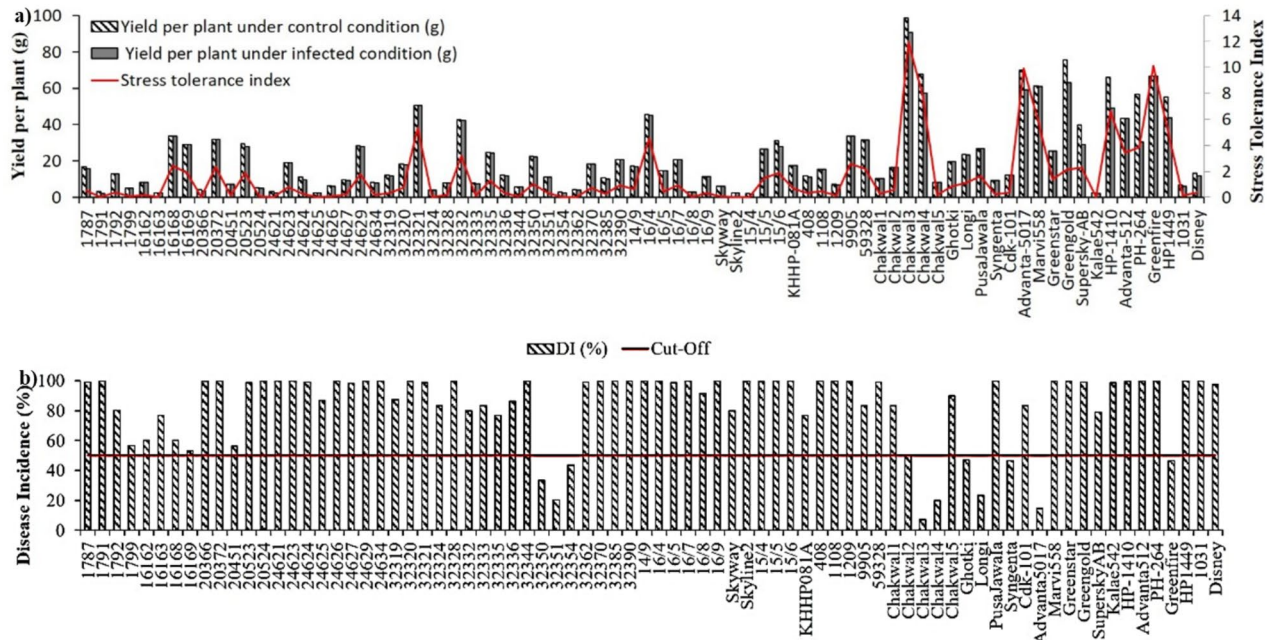
Fig. 1. (a) Bar graphs are depicting yield per plant-YPP (g) in the control and in the infected conditions, however, red line is showing stress tolerance index-STI for *Phytophthora capsici* root rot in chilli and (b) is showing disease incidence-DI (%) based on resistance level scale whereas red line is showing the threshold level (50%) between resistance and susceptibility for *Phytophthora capsici* root rot in chilli.

adjusted values of chilli genotypes, the superior check Chakwal3 was found best performing for yield per plant under the infected condition (mean $_{check}$+LSI $_{check}$=90.70 g).

Stress tolerance index

The analysis of variance was observed with significant mean sum of square for stress tolerance index is shown in Table 1. The range of STI values were observed from 0.01 (16163) to 11.98 (Chakwal3) with mean value of 2.2. Moreover, the comparison of adjusted values of chilli genotypes with LSI based value of checks found that best performing check Chakwal3 has maximum STI (mean $_{check}$+LSI $_{check}$ =12.12) than other genotypes (Supplementary Table S5 online).

Relative cell injury

The Table 1 shows the analysis of variance for relative cell injury, that has the significant mean sum of square. Moreover, the minimum RCI was observed in genotype Greenfire with 23.16% whereas Advanta512 was found with maximum cell injury i.e. 88.56% with mean of 66.06% value (Fig. 2a). Plants detected with low relative cell injury under stress resulted in higher yield and termed as best performer. Therefore, based on LSI test, adjusted values of all genotypes exceeded from superior check Chakwal3 that confirmed the presence of lowest RCI (mean $_{check}$+LSI $_{check}$=28.11%) in Chakwal3 (Supplementary Table S5 online).

Cell viability

The analysis of variance in Table 1, depicted the significant mean sum of square for cell viability. The highest CV of 93.96% was recorded for genotype Greenfire whereas 32,344 was found with minimum CV i.e. 16.11% with mean value of 52.34% (Fig. 2b). The LSI based value of checks revealed that the adjusted value of genotype Greenfire (94.17%) was exceeded from superior check Chakwal3 (mean $_{check}$+LSI $_{check}$=93.31%) (Supplementary Table S5 online).

Disease incidence

The analysis of variance was observed with significant mean sum of square for disease incidence is shown in Table 1. Moreover, the minimum DI of 6.81% was shown in genotype Chakwal3 whereas twenty-four numbers of genotypes were found with maximum DI of 100% with mean value of 78.66% (Table 1). A plant with reduced disease incidence percentage was considered to be healthier. Therefore, based on LSI test the check Chakwal3 was found superior with lowest DI (mean $_{check}$+LSI $_{check}$=7.17%), then adjusted values of all chilli genotypes (Supplementary Table S5 online).

Disease severity index

The analysis of variance with significant mean sum of square for disease severity index (%) is shown in Table 1. The genotype Chakwal3 was found with maximum DSI i.e. 1.12% whereas four genotypes i.e. 24,629, 32,390,
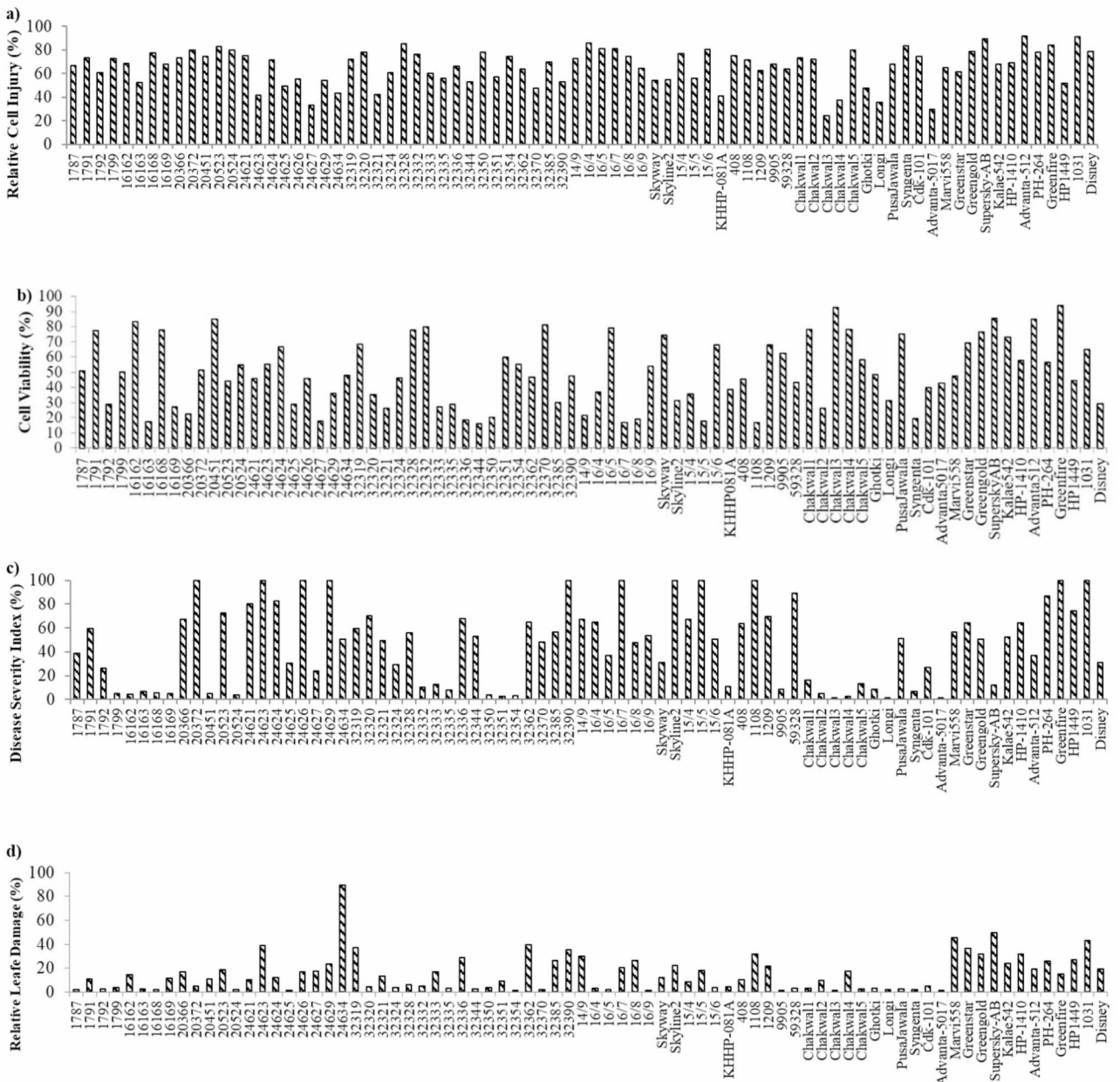
Fig. 2. **(a)** Relative cell injury-RCI (%), **(b)** cell viability-CV (%), **(c)** disease severity index-DSI (%) and **(d)** relative leaf damage-RLD (%) for *Phytophthora capsici* root rot in chilli.

Skyline 2 and 1108, were observed with 100% DSI with mean value of 44.77% (Table 1; Fig. 2c). Plants detected with low DSI under stress condition considered as healthier plants. Therefore, the comparison of LSI based mean of checks with adjusted values of all genotypes detected that genotype Advanta5017 was found better for DSI trait (1.82%) than superior check Chakwal3 (mean $_{check}$+LSI $_{check}$=2.01%) (Supplementary Table S5 online).

Relative leaf damage

The analysis of variance has shown the significant mean sum of square for relative leaf damage (%) that is given in Table 1. RLD value was observed ranges from 1.10% (32354) to 89.45% (24634) with mean value of 15.22% (Table 1 Fig. 2d). Low percentage of RLD in plants under stress resulted in healthier plants. Therefore, the adjusted values of four genotypes i.e. 32,354 (0.57%), Advanta5017 (0.57%), 24,625 (0.67%) and Longi (0.74%) exceeded from the LSI based mean value of superior check Chakwal3 for trait RLD (mean $_{check}$+LSI $_{check}$=0.90%) (Supplementary Table S5 online).

Selection of resistant germplasm

The resistance level scale classified 3 genotypes (Chakwal3, Advanta5017 and Chakwal4) as resistant: R, followed by 7 genotypes (32351, Longi, 32350, 32354, Syngenta, Ghotki and Greenfire) as moderately resistant: MR and the rest of 68 genotypes as susceptible: S (Fig. 1-b, Supplementary Table S6 online).

Principal component analysis (PCA)

The data of phenotypic traits were subjected to principal component analysis (PCA) that resulted with maximum variance for PC1 and PC2 (42.8% and 25.30%, respectively) contributing 68.08% of cumulative variance (Supplementary Table S6 online) as evident from scree plot (Fig. 3-a). The traits having component loading value of 0.5 or more are considered as major contributors. Therefore, the major contribution was observed by STI with 0.86 value for PC1 whereas DI for PC2 with 0.71 value is given in Supplementary Table S6. The PCA biplot (Fig. 3-b) was generated on basis of first two principal components to detect the significant chilli genotypes and the relationship among all measured traits. The biplot (Fig. 3-b) showed that genotypes Chakwal3, Chakwal4, Advanta5017, and Greenfire were found far from the central point that depicts the significance of these genotypes in the current germplasm collection. Conversely, the vectors on PCA biplot with acute angle show positive correlation, obtuse angle show negative correlation while right angle shows zero correlation among trait. Therefore, the biplot depicts the close correlation of traits RLD, DI, DSI and RLD and are oppositely correlated with traits YPPc, YPPi, STI and CV (Fig. 3-c and -b). The Fig. 3-c shows the presence of genotypes in their respective ellipses: Resistant, moderately resistant and susceptible on basis of resistance level scale (Supplementary Table S5 online). It clearly separated resistant genotypes from moderately resistant and susceptible genotypes. Saturation of genotypes was found in ellipses for susceptible genotypes.

Molecular assessment

The previously detected thirty-four PcRR associated SSR markers[22] were amplified for seventy-eight chilli genotypes in order to find significant SSRs that leads to successful marker assisted selection.
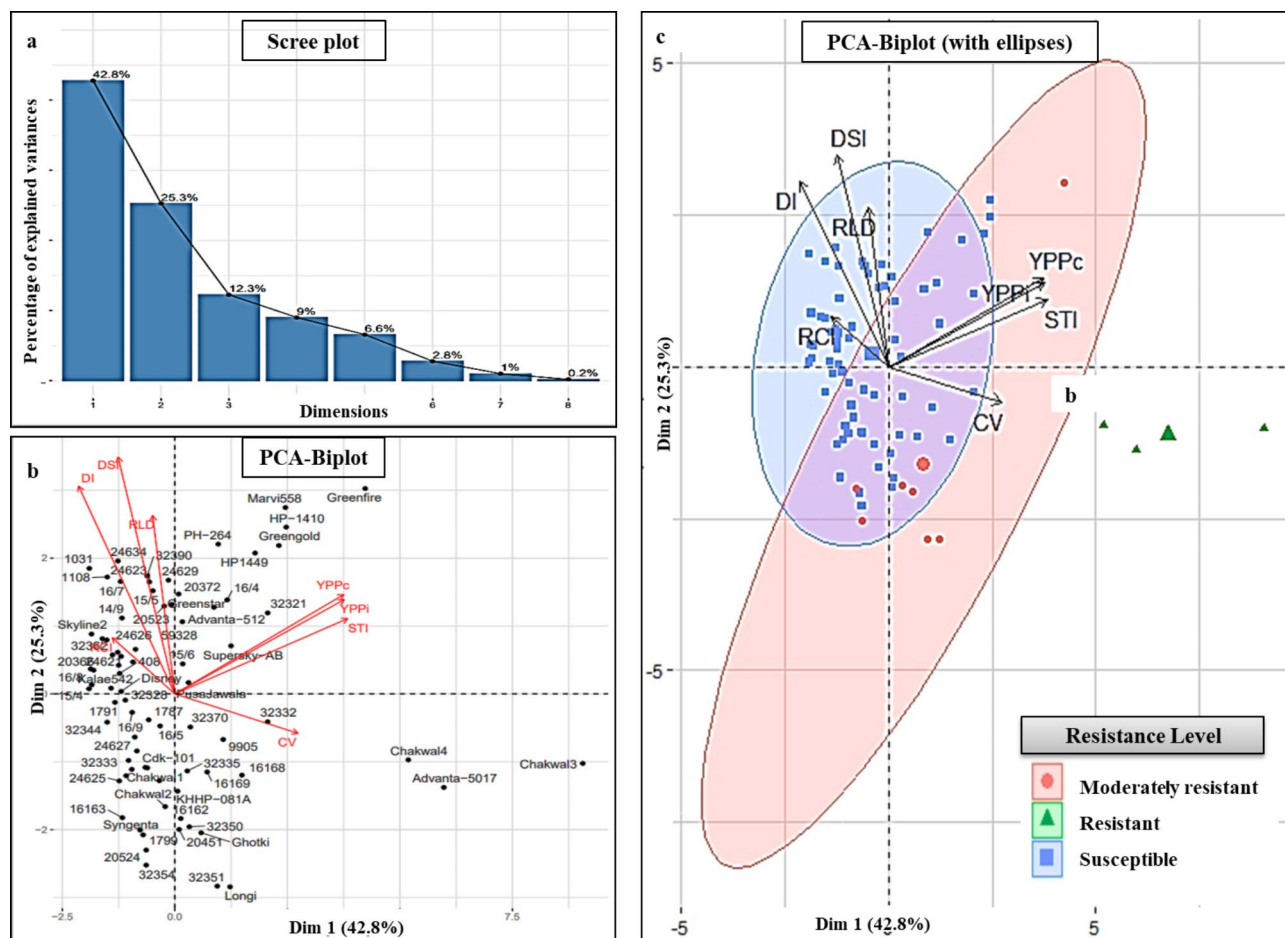

Fig. 3. Scree plot **(a)**, Biplot **(b)**, showing distribution of genotypes and traits whereas biplot **(c)**, showing ellipses for three resistance levels in chilli germplasm.

Markers	*P*-value	Associated traits
Hpms1172	0.0003*	Stress tolerance index
CAMS177	0.0465*	Stress tolerance index
CAMS066	0.0435*	Relative cell injury
CA06g27450	0.0003*	Disease incidence
CAMS173	0.0001*	Relative leaf damage
*=significant		

Table 2. List of validated SSR markers along with their associated phenotypic traits.
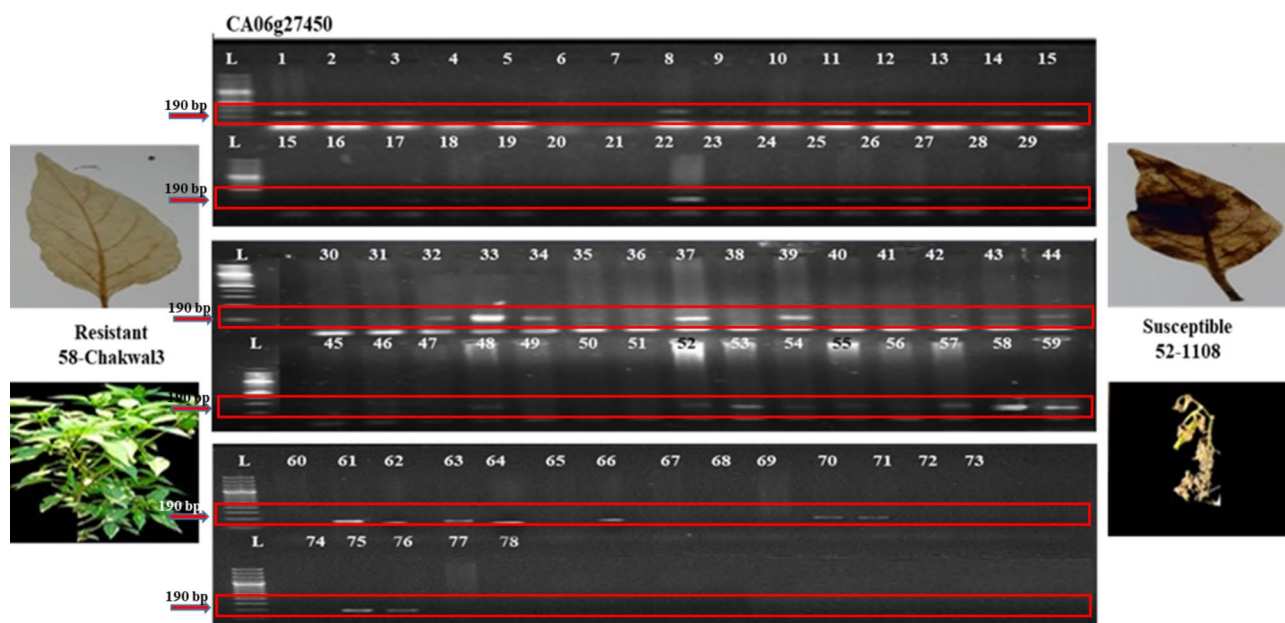

Fig. 4. The gel picture of significant marker CA06g27450 associated with disease incidence showing visualization of bands for resistant and susceptible chilli genotypes along with pixels of their respective plants and H$_2$O$_2$ accumulated DAB stained leaves.

Validation of markers

The means for traits of 78 genotypes among 34 SSR markers were grouped by comparing them on basis of binary data i.e. 1 for presence and 0 for absence of the marker using student's *t*-test. The student's *t*-test detected five significant trait specific markers out of thirty-four markers. The SSR markers. Hpms1172 and CAMS177 were found significant for stress tolerance index with 0.0003 and 0.0465 P-value, respectively. Whereas, marker CAMS066 with 0.0435 P-value for relative cell injury, CA06g27450 with 0.0003 P-value for disease incidence and CAMS173 with 0.0001 P-value for relative leaf damage, were found significant (Table 2). The Fig. 4 is the gel picture of marker CA06g27450 found significant for disease incidence shows the presence of bright band for resistant genotype i.e. Chakwal3 as well as absence of bands for susceptible chilli genotype i.e. 1108 (Table 2). Moreover, the Table 3 showed that maximum genotypes were found associated for markers CAMS177, CAMS066, CA06g27450 and CAMS173 whereas eight genotypes were found associated with marker Hpms1172.

Marker assisted selection (MAS)

The significant trait specific markers were further leads to marker assisted selection by appearance of bright bands on the gel pixels for chilli genotypes (Supplementary Figure. S1a-e online). The gel picture of marker Hpms1172 has produced clear bands at 200 bp size for genotypes, Chakwal3, Greenfire, Greengold, Advanta5017, Chakwal4, 32,321, HP1410 and 16/4 (Supplementary Figure. S1-a online), whereas marker CAMS177 has shown clear bands at 150 bp size for genotypes, Chakwal3, Greenfire, Greengold, Advanta5017, Chakwal4, 32,321, HP1410, 16/4, Marvi558 and HP1449 (Supplementary Figure. S1-b online). Moreover, the gel picture of SSR markers viz. CAMS066, CA06g27450 and CAMS173 showed the appearance of bright bands at 290 bp, 190 bp and 210 bp of size, respectively for chilli genotypes, Chakwal3, Chakwal4, Longi, Advanta5017, 32,350, 32,351, 32,354, Ghotki, Syngenta and Greenfire (Supplementary Figure. S1c-e online).

Moreover, principal coordinate analysis (PCoA) was carried out to further validate the obtained data for MAS. Principal coordinate analysis (PCoA) plot confirmed the genetic relatedness and discrete grouping that yielded into significantly distinct populations (resistant, moderately resistant and susceptible) on Fig. 5. The

Genotype		Hpms1172	CAMS177	CAMS066	CA06g27450	CAMS173
S#	Size (bp)	200	150	290	190	210
1	1787	-	-	-	-	-
2	1791	-	-	-	-	-
3	1792	-	-	-	-	-
4	1799	-	-	-	-	-
5	16,162	-	-	-	-	-
6	16,163	-	-	-	-	-
7	16,168	-	-	-	-	-
8	16,169	-	-	-	-	-
9	20,366	-	-	-	-	-
10	20,372	-	-	-	-	-
11	20,451	-	-	-	-	-
12	20,523	-	-	-	-	-
13	20,524	-	-	-	-	-
14	24,621	-	-	-	-	-
15	24,623	-	-	-	-	-
16	24,624	-	-	-	-	-
17	24,625	-	-	-	-	-
18	24,626	-	-	-	-	-
19	24,627	-	-	-	-	-
20	24,629	-	-	-	-	-
21	24,634	-	-	-	-	-
22	32,319	-	-	-	-	-
23	32,320	-	-	-	-	-
24	32,321	+	+	-	-	-
25	32,324	-	-	-	-	-
26	32,328	-	-	-	-	-
27	32,332	-	-	-	-	-
28	32,333	-	-	-	-	-
29	32,335	-	-	-	-	-
30	32,336	-	-	-	-	-
31	32,344	-	-	-	-	-
32	32,350	-	-	+	+	+
33	32,351	-	-	+	+	+
34	32,354	-	-	+	+	+
35	32,362	-	-	-	-	-
36	32,370	-	-	-	-	-
37	32,385	-	-	-	-	-
38	32,390	-	-	-	-	-

Genotype		Hpms1172	CAMS177	CAMS066	CA06g27450	CAMS173
S#	Size (bp)	200	150	290	190	210
41	16/5	-	-	-	-	-
42	16/7	-	-	-	-	-
43	16/8	-	-	-	-	-
44	16/9	-	-	-	-	-
45	Skyway	-	-	-	-	-
46	Skyline2	-	-	-	-	-
47	15/4	-	-	-	-	-
48	15/5	-	-	-	-	-
49	15/6	-	-	-	-	-
50	KHHP081A	-	-	-	-	-
51	408	-	-	-	-	-
52	1108	-	-	-	-	-
53	1209	-	-	-	-	-
54	9905	-	-	-	-	-
55	59,328	-	-	-	-	-
56	Chakwal1	-	-	-	-	-
57	Chakwal2	-	-	-	-	-
58	Chakwal3	+	+	+	+	+
59	Chakwal4	+	+	+	+	+
60	Chakwal5	-	-	-	-	-
61	Ghotki	-	-	+	+	+
62	Longi	-	-	+	+	+
63	PusaJawala	-	-	-	-	-
64	Syngenta	-	-	+	+	+
65	Cdk-101	-	-	-	-	-
66	Advanta5017	+	+	+	+	+
67	Marvi558	-	+	-	-	-
68	Greenstar	-	-	-	-	-
69	Greengold	+	+	-	-	-
70	Supersky-AB	-	-	-	-	-
71	Kalae542	-	-	-	-	-
72	HP-1410	+	+	-	-	-
73	Advanta-512	-	-	-	-	-
74	PH-264	-	-	-	-	-
75	Greenfire	+	+	+	+	+
76	HP1449	-	+	-	-	-
77	1031	-	-	-	-	-
78	Disney	-	-	-	-	-

Continued

S#	Genotype Size (bp)	Hpms1172 200	CAMS177 150	CAMS066 290	CA06g27450 190	CAMS173 210
1	1787	-	-	-	-	-
39	14/9	-	-	-	-	-
40	16/4	+	+	-	-	-

S#	Genotype Size (bp)	Hpms1172 200	CAMS177 150	CAMS066 290	CA06g27450 190	CAMS173 210
41	16/5	-	-	-	-	-
Total present		08	10	10	10	10
Total absent		70	68	68	68	68

Table 3. Amplification of Chilli genotypes at the 5 validated SSR markers by showing '+' sign for the presence of bands whereas '–' sign for the absence.
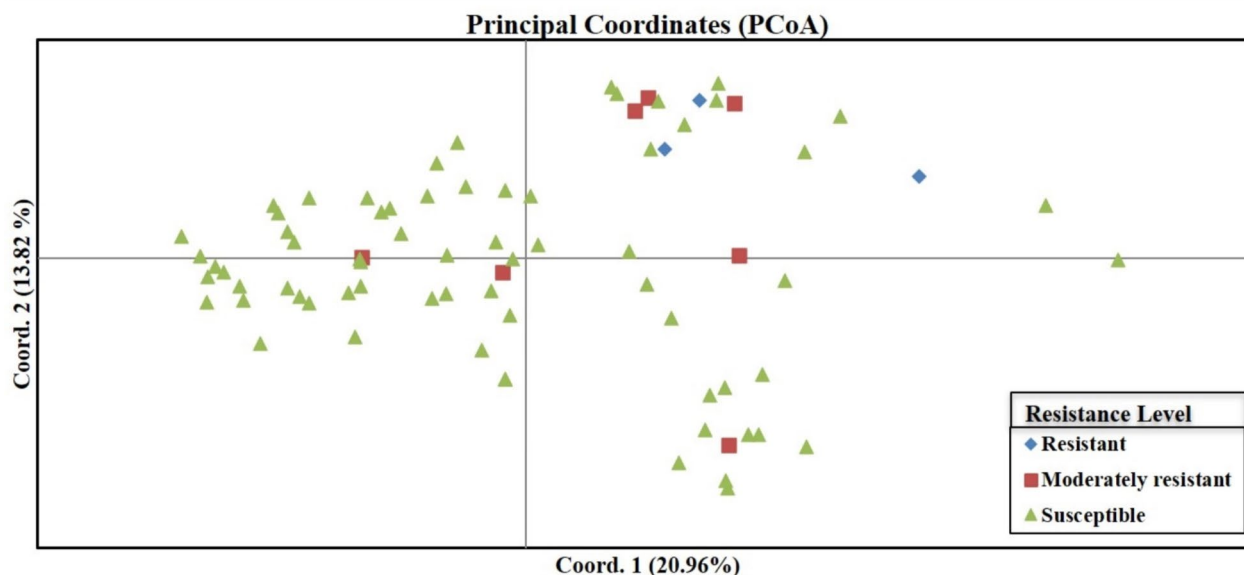
8

Fig. 5. Principal coordinate analysis (PCoA) of chilli germplasm based on data of SSR markers.

first coordinate has shown 20.96% whereas second coordinate explained 13.82% of the variation with 34.78% of cumulative variance for both coordinates. The major contribution was observed by genotype Chakwal3 for both PCoA 1 and PCoA 2 with 0.97 and 0.48 values, respectively (Supplementary Table S7 online).

Discussion

Phytophthora capsici root rot (PcRR) is one of the disastrous oomycetes, which causes drastic reduction in yield and productivity of chilli[24]. It is considered as a notorious pathogen that could cause damages from 30–100%[25]. Despite of the destructive nature of the disease, it is not controllable due to cultural practices. The fungicides are mostly used on infected fields for *Phytophthora* root rot management that resulted in development of fungicide resistance in crop that leads to environmental hazards. Moreover, presence of different *Phytophthora* races severely complicates the control of this pathogen. It requires keen attention of plant breeders to screen and detect PcRR resistant chilli genotypes which are phenotypically superior and are able to achieve sustainable production. Therefore, present study was designed for marker assisted selection and validation of previously reported 34 SSR markers using 78 chilli genotypes[23].

A series of experiments were conducted to achieve the planned tasks to screen out PcRR resistant chilli genotypes having high yield tendency from a germplasm collection under stress (PcRR infected sick bed)[26]. Crop yield is the main target of plant breeders to improve the productivity and quality of their crop under stress[27,28]. Phenotypic assessment was majorly dependent on the screening of germplasm for disease resistance[29]. The superior genotypes with ability of disease resistance along with high yield were identified by selecting genotypes with maximum stress tolerance index[30,31]. Therefore, in current study the genotype Chakwal3 was found as best due to existence of maximum yield under both control:98.61 g and infected condition:90.69 g resulted in maximum stress tolerance index:11.98 value (Fig. 1-a; Supplementary Table S4 online). Thus, stress tolerance index is mostly used to differentiate between resistant and susceptible genotypes[32,33]. Moreover, the genotypes Greenfire, Advanta-5017 and Chakwal4 were also found best due to increased STI of 10.14, 9.94 and 7.8 value, respectively. It was noted that there was lack of significant findings in the quest to identify superior genotypes through stress tolerance index analysis in chilli, for both biotic and abiotic stresses. The STI was obtained in the study of Aktar-Uz-Zaman et al., to get desirable genotypes for high temperature condition in lentil crop, whereas, in the study of Mudi, Mahapatra, & Das, stable genotypes were identified for the assessment of *Helminthosporium* blight resistance in barley[30,34].

Plants that exhibit resistant to disease have low percentage of disease incidence, which results in improvement of its health and growth. This improvement is supported by a healthy root system that enhances plant resistance, ultimately resulting in increased crop yield[35]. Therefore, the genotype Chakwal3 have shown reduced disease incidence (6.81%) and disease severity index (1.12%). Whereas, the genotype Greenfire was observed with minimum relative cell injury (23.16%) and maximum cell viability (93.96%). Our currently obtained results contribute more to useful physiological features in chilli that have been connected to PcRR resistance, which can be used as the platform for detecting resistant genotypes that can be integrated into agricultural system for crop improvement. On basis of scale proposed by Jo et al., ten genotypes including three resistant genotypes i.e. Chakwal3, Advanta5017, Chakwal4 and seven moderately resistant genotypes i.e., 32,351, Longi, 32,350, 32,354, Syngenta, Ghotki and Greenfire[36](Figure. 1-b; Supplementary Table S5 online).

In current study the multivariate analysis or principal component analysis (PCA) helped to evaluate the significant genotypes for PcRR resistance using various traits and also showed the interrelation among these

traits[37,38]. The Fig. 3-b depicted the PCA biplot based on PC1 and PC2 confirmed the significance of genotypes Chakwal3, Chakwal4, Advanta-5017 and Greenfire in the current study by finding them away from the central point. Therefore, these genotypes were also observed as major contributors[39]. According to Akand et al., the vectors in PCA biplot with acute angle show positive correlation, obtuse angle show negative correlation while right angle show zero correlation among trait[40]. Therefore, Fig. 3b-c confirmed the obtained findings by the existence of significantly positive association of STI with the yield per plant under both the control and the infected conditions. Moreover, the currently analyzed PCA showed that traits STI and CV are oppositely correlated with RCI, RLD, DI and DSI traits that ease the selection of compatible disease resistant genotypes from chilli germplasm[41,42].

Numerous genes were discovered that encodes the regulation of disease resistance and susceptibility in plants by detecting pathogen invasion through signalling the plant components for sudden defense reaction[43,44]. The resistance mechanism in chilli plants is due to presence of R genes that are able to control 45 different *Phytophthora capsici* races[45]. Therefore, using conventional breeding methods for PcRR resistance in chilli is complicated and challenging that invokes the use of marker assisted selection for incorporating PcRR resistance in chilli[46]. However, marker identification followed by validation is critical prerequisite in the current study. Here, in the current study the previously identified 34 SSR markers were amplified using seventy-eight chilli genotypes and were validated to get trait specific significant markers[23] (Table 2). Currently, the validation studies have obtained of 34 SSR markers that resulted in detection of five significant trait specific SSR markers. Therefore, trait specific markers including Hpms1172, CAMS177, CAMS066, CA06g27450 and CAMS173, whereas, Kumar, Kambham, Reddy, Sriram, & Singh reported one SSR marker (HpmsE034), Bongiorno et al., reported one SNP-based CAPS marker (*Phyto* CAPS), Zhang et al. reported seven KASP markers and Moreira et al. reported AFLP markers (Mcaa14/Eacg, Mcaa15/ Eacg) for PcRR resistance in chilli[18,47–49]. Interestingly, in the present study the both the SSR markers i.e. Hpms1172 and CAMS177 has shown significance for stress tolerance index, Moreover, the current finding will be helpful in enhancement of crop productivity, that is dependent on disease resistance in all crops[50]. In accordance with present results the study of Pawar et al. the SSR marker RM302 was found significant for days to 50% flowering as well as for days to maturity trait[51]. Moreover, the validation studies of Zeng, Meredith, Gutiérrez, & Boykin depicted that marker BNL285 was found associated with traits lint percentage and boll weight in cotton crop[52]. Moreover, the significant chilli genotypes that reside in these markers can be used in MAS for enhancement of yield as well as for disease resistance. The significant trait specific association of markers was also obtained in Pea and rice using the student's *t* test[51,53]. The associated markers were considered as most reliable markers to utilize in crop improvement[54]. Therefore, the validation of trait specific markers is an important step to precede marker assisted selection[49].

Moreover, current validation of markers confirmed the significance of commonly identified genotypes, Chakwal3, Greenfire, Advanta5017 and Chakwal4 for all five markers (Table 3; Supplementary Table S7 online; Figs. 4 and 5; Supplementary Figure. S1a-e online). Many commercial varieties are either partially resistant or even highly susceptible to *Phytophthora* root rot. Therefore, the identification of trait specific markers for PcRR resistance would be helpful for breeders to detect resistant genotypes in shortest time[55]. Previously, Kumar, Kambham, Reddy, Sriram, & Singh reported two, whereas, Wang, Wang, Guo, Yang, & Shen identified one genotype through marker assisted selection (MAS) for PcRR resistance in chilli[13,18]. The detected resistant genotypes were also found to be high yielding (Fig. 1-a; Supplementary Table S5 online). The accuracy of previously detected markers significantly shows variations if used for different chilli germplasms for disease related traits as observed in the study of Siddique et al., where 117 SNP marker associated with PcRR resistance were identified[56]. This variation in phenotype-genotype interaction is an important obstruction that limits the efficacy of MAS, making it challenging for PcRR resistance. The detected markers for PcRR resistance will lay the foundation for the molecular profiling of PcRR resistance in future breeding programmes. Therefore, there is dire need of continuous practice for detecting resistant genotypes through marker assisted selection (MAS) for PcRR resistance in chilli.

Conclusion

Phytophthora capsici root rot (PcRR) is a destructive oomycete that difficult to control due to its polygenic nature. Therefore, the present study was planned for validation of previously reported markers to detect significant trait specific markers as well as marker assisted selection of PcRR resistant chilli genotypes. Furthermore, the validation of 34 previously detected SSR markers resulted in five significant trait specific markers. Furthermore, the association of SSR marker CAMS066 was observed with trait RCI, CA06g27450 with trait DI and CAMS173 with trait RLD. However, both the SSR markers Hpms1172 and CAMS177 were found associated with trait STI. The marker assisted selection confirmed the significance of phenotypically detected superior genotypes (Chakwal3, Greenfire, Advanta5017 and Chakwal4) by appearance of bright bands on gel pictures. The obtained marker assisted selection facilitates the early detection of high yielding resistant genotypes against drastic pathogen *Phytophthora capsici*. Therefore, the obtained significant SSR markers and marker assisted selection of chilli genotypes would be helpful in incorporating PcRR resistance in chilli hybrids for future breeding programs.

Materials and methods
Nursery preparation and experiment layout

The germplasm of 78 chilli genotypes was obtained from Plant Genetic Resource Centre (PGRI), National Agriculture Research Centre (NARC), Islamabad, Pakistan (Supplementary Table S1 online). The collected seeds were haloprimed by soaking chilli seeds in 3% KNO_3 solution for 24 h to enhance germination ability of seeds[57]. Moreover, primed seeds were sown in trays filled with coco-peat in March 2020 (all experimental studies and experimental materials involved in this research are in full compliance with relevant institutional,

national and international guidelines and legislation). Whereas, in May 2020, the two sets of chilli seedlings were transplanted at Plant Pathology section, Agriculture Research Institute Mingora, Swat, Pakistan (34.77 °N-72.34 °E). Each set consists of all 78 chilli genotypes (10 plants/genotype). One set of chilli seedlings was transplanted in field as the control condition, whereas second set was transplanted on PcRR infected sick bed as the infected condition. *Phytophthora capsici* root rot (PcRR) infected sick bed was prepared and maintained according to Shaw et al.[25]. Plants were transplanted with 60 cm of row to row distance and 45 cm of plant to plant distance in augmented block design (ABD) having four blocks. Nineteen chilli genotypes along with two checks were placed in each block. Cultural practices like irrigation, hoeing, roughing of weeds and application of NPK fertilizer etc. were applied as per recommendations. The crop was fertilized with N: P:K (20:20:20) solution in split doses. Furthermore, the screening and phenotyping was practiced on raised crop.

Phenotyping

Disease scoring When symptoms started appearing on plants, the scoring was observed on basis of disease scoring table (Supplementary Table S2 online) proposed by Bosland & Lindsey[58] with few modifications to find out disease incidence (DI) % and disease severity index (DSI) % according to Aklilu, Ayana, Abebie, & Abdissa[59],

$$Disease\ incidence\,(DI)\,\% = \frac{Number\ of\ infected\ plants\ per\ row}{Total\ number\ of\ plants\ per\ variety} \times 100$$

$$Disease\ severity\ index\,(DSI)\,\% = \frac{[(0 \times a) + (1 \times b) + (2 \times c) + (3 \times d) + (4 \times e) + (5 \times f)]}{[(a + b + c + d + e + f) \times 5]} \times 100$$

where a, b, c, d, e and f refer to the numbers of plants fall under each score of scoring scale (0 to 5) related to their respective disease symptoms. The selection of genotypes was subjected to the model proposed by Jo et al. for classification of resistance level[36]. The genotype was considered as R: resistant if DI < 20%, MR: moderately resistant if DI = 20–50% and S: susceptible if DI > 50%.

Stress tolerance index (STI) Yield per plant (YPP) of all the chilli genotypes was recorded for both the control as well as for the infected conditions. Moreover, on basis of yield data, stress tolerance index (STI) was calculated using formula proposed by Fernandez[60],

$$Stress\ tolerance\ index\,(STI) = (YPP_c \times YPP_i)/YPPC^2$$

Where, YPP_c = yield of genotype under control condition, YPP_i = yield of genotype under infected condition and YPPC = mean yield of all genotype under control condition. A higher STI value for any genotype indicates its greater ability to tolerate disease and potentially achieve a higher yield.

Relative cell injury (%) Relative cell injury (RCI) % was determined on the basis of electrolyte leakage following the modified method devised by Widmer, Graham, & Mitchell[61]. Initially, a set of ten root tips (15 mm) of each genotype from the control and the infected plants were collected. Each set of root tips were washed thrice in sterile, double-distilled water to remove any residual electrolytes and were placed in a test tube containing 15 ml of sterile, deionized distilled water. The samples were placed at room temperature for 24 h and the conductivity of the solution was measured using Electric Conductivity Meter (EC 215, Hanna Instruments). All test tubes were then sealed with cotton swabs and autoclaved for 15 min at 121 °C temperature to kill all cells. Samples were equilibrated to room temperature to take final conductivity.

The electrolyte leakage percentage was measured using following functions:

$$Electrolyte\ Leakage(EL)_{control}\% = 1 - C_1/C_2 \times 100,$$

$$Electrolyte\ Leakage(EL)_{infected}\% = 1 - T_1/T_2 \times 100,$$

Furthermore, relative cell injury (%) was calculated by using EL (%) value by following the formula proposed by Nijabat[62].

$$Relative\ cell\ injury\,(RCI)\,\% = 1 - (EL_{infected}\%/EL_{control}\%) \times 100,$$

whereas, $C_1 = EL_{control}$% before autoclave, $C_2 = EL_{control}$% after autoclave, $T_1 = EL_{infected}$% before autoclave, $T_2 = EL_{infected}$% after autoclave.

Cell viability (%) For measuring cell viability (CV) %, ten root tips from each observation per genotype (3 observations/genotype) were obtained from plants of the control condition as well as from the infected condition separately. The collected samples were washed with sterile, double-distilled water and were subjected to Evans blue aqueous solution (dye) with the volume of 0.25% (v/v) for 15 min, as per method proposed by Hameed, Keitel, Ahmad, Mahmood, & Trethowan[63]. The dyed root tips were then extensively washed separately with distilled water for 30 min to remove excess and unbound dye. The bounded dye to the dead cells was then solubilized in 50% (v/v) ethanol with 1% (w/v) SDS (Sodium Dodecyl Sulfate) at 60 °C for 30 min. The optical density (OD) was quantified by measuring absorbance at 600 nm from Spectrophotometer[64]. Another set of root tips from both the conditions was subjected to autoclave for killing cells, then treated with dye and latterly OD was measured. Cell viability was calculated using following formula.

$$Cell\ viability\,(CV)\,\% = \frac{Infected\,(OD_{autoclaved} - OD_{non-autoclved})}{Control\,(OD_{autoclaved} - OD_{non-autoclved})} \times 100$$

Relative leaf damage (%) The relative leaf damage (RLD) % was determined by detecting Hydrogen Peroxide (H_2O_2) by using DAB (3,3'-Diaminobenzidine) staining method as proposed by Daudi & O'Brien[65]. Young growing leaves (3 leaves/plant) of control/infected chilli plants were placed in 6 well micro titre plates, separately. Two ml of the Na_2HPO_4 DAB staining solution was poured in plates containing sample leaves. Moreover, solution from microtitre plates was discarded through vacuum infiltration, by placing plates in a dessicator for 5 min. Plates were then wrapped with aluminum foil and were then placed on a shaker for 4–5 h at 80–100 rpm speed. After incubation, foil was removed and solution was replaced by bleaching solution (ethanol: acetic acid: glycerol; 3:1:1). Chlorophyll on leaves was bleached by placing plates in a boiling water bath (90–95 °C) for 15 min. Formation of brown precipitates in plates having inoculated leaves was due to DAB reaction with the hydrogen peroxide. Few precipitates were found in plates with un-inoculated leaves. The stains on leaves were captured by placing treated leaves on white surface under uniform light. The total leaf area and DAB-stained leaf area of chilli leaves from both the control and the infected conditions was obtained by uploading photographs of DAB-stained leaves in Image J. software. The percent leaf area for both control and infected condition was calculated using formula proposed by Li et al.[66].

$$Percent\ leaf\ area_{infected} = DAB\ stained\ leaf\ area/total\ leaf\ area \times 100$$

$$Percent\ leaf\ area_{control} = DAB\ stained\ leaf\ area/total\ leaf\ area \times 100$$

Furthermore, relative leaf damage % was calculated by using following formula:

$$Relative\ leaf\ damage\,(RLD)\,\% = Percent\ leaf\ area_{infected}/Percent\ leaf\ area_{control} \times 100$$

Data analysis for phenotypic traits Collected phenotypic data was statistically computed using R (version: 1.3.1093) computer software[67]. The agricolae R package was used for analysis of variance (ANOVA) according to d Steel & Torrie[68], and least significant increase (LSI) was obtained according to Federer & Raghavarao[69] for Augmented Block Design (ABD). LSI was added to the mean value of each check and thus resulted value of superior check was compared with the adjusted values of other genotypes. The genotype better from superior check (mean $_{check}$ +LSI $_{check}$) with maximum value was termed as best performing genotype among the studied germplasm. Furthermore, FactoMineR R package was used for determining principal component analysis (PCA) and constructing PCA-biplot with or without ellipses.

Validation of markers and marker assisted selection (MAS)

DNA extraction and molecular profiling Genomic DNA of 78 chilli genotypes was extracted according to the CTAB (cetyl trimethylammonium bromide) procedure proposed by Doyle[70]. The concentration and purity were assessed by observing absorbance ratio at 260:280 nm with a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Germany) using 1 µL of each sample. The DNA of all 78 chilli genotypes were amplified using previously identified 34 polymorphic SSRs[23] (Supplementary Table S3 online). PCR analysis was carried out in a reaction volume of 25 µL using method proposed by McGregor et al.[71]. The reaction mixture contained 1 ng template DNA, 0.2 U Taq polymerase, 0.4 µM of forward primer, 0.4 µM of reverse primer and 0.4 mM dNTPs. DNA amplification was carried out in the thermal cycler (T100, BIORAD, USA). PCR was set with 1-minute initial denaturation, followed by 35 cycles of denaturation at 95 °C for 25 s, annealing (specified for each primer for 25 s) and extension at 72 °C for 30 s. The reaction was completed by a final extension at 72 °C for 15 min. Samples were kept at hold at 4 °C after the final step. The amplified fragments were run along with 1 kb DNA ladder (Thermofisher), on 1.5% agarose gel and observed under Gel Documentation System Syngene (Model: InGenius3) and bands were recorded accordingly. The appearance of visually bright bands on gel pictures were considered as present, whereas considered as absent in case of absence of bands.

Statistical analysis for validation of markers All markers were individually analyzed by using Student's t-test[72] as reported by Pawar, Suresh, Hittalmani, BC, & Biradar[51].

$$Sp^2 = \frac{S_1^{\,2}\,(n_1 - 1) + S_2^{\,2}\,(n_2 - 1)}{n_1 + n_2 - 2}$$

$$Student's\,t-test = \overline{X}_1 - \overline{X}_2/\sqrt{\left(Sp^2\,[1/n_1 + 1/n_2]\right)}$$

$\overline{X}_1$ = mean of trait that reside under genotypic value 1, $\overline{X}_2$ = mean of trait that reside under genotypic value 0, Sp^2 = pooled variance, n_1 = number of genotypes in $\overline{X}_2$ and n_2 = number of genotypes in $\overline{X}_2$. Moreover, the data was considered as significant on basis of threshold values i.e. P-value ($P < 0.05$).

Principal coordinate analysis Furthermore, principal coordinate analysis (PCoA) was computed using GenAlex software[73].

Data availability
All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Author contributions

Conceptualization, T.B. and R.M.R.; methodology, A.I.K, M.A.K., and A.U.; formal analysis, M.N. and R.I; investigation, H.R., M.S.E., M.R., R.I.; writing—original draft preparation, T.B.; writing—review and editing, All authors.; visualization, R.M.R.; supervision, T.B. and R.M.R.; All authors have read and agreed to the published version of the manuscript.

Declarations

Ethics approval and consent to participate
This study does not include human or animal subjects.

Consent for publication
Not applicable.

Competing interests
The authors stated that they had no interest which might be perceived asposing a conflict or bias.

Statement on guidelines
All experimental studies and experimental materials involved in this research are in full compliance with relevant institutional, national and international guidelines and legislation.

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Correspondence and requests for materials should be addressed to R.M.R., M.R. or R.I.

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